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Original Basic Science Studies

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Background

Hypoxemic ischemic encephalopathy (HIE) is a major cause of morbidity and mortality in the neonatal population. Recent research has shown human adipose stem cells (hASCs) to have neuroprotective effects in animal models of HIE. This study tested the hypothesis that neurodevelopmental outcomes would be improved using hASC therapy in term neonatal HIE rat model.

Methods

Seven day old rats underwent left carotid artery ligation followed by 8% oxygen exposure for 120 minutes, or carotid isolation in shams. Forty-eight hours after surgery half of the rats received hASCs and half normal saline (NS) intravenously. Rota-rod and cylinder tests were used to assess skill learning, balance, coordination, and symmetry of limb use at 2, 4 and 6 weeks of life.

Results

HIE rats treated with hASCs traveled further on Rota-rod (p=0.03) when compared to HIE with NS groups. Those in the HIE-hASC group had significant improvement in the usage of the affected limb at week 6 (p=0.03) compared to those with HIE and NS. Rats receiving hASCs post HIE had less cortical atrophy compared to those with HIE and NS.

Conclusions

Rats with HIE treated with hASCs showed improvement in long-term neurodevelopmental aspects and decreased cortical atrophy compared to HIE control group.

INTRODUCTION

Hypoxic ischemic encephalopathy (HIE) is a leading cause of perinatal mortality and severe neurologic impairment worldwide.[1] The incidence is approximately 8 per 1000 term births, with a mortality rate as high as 60% in severe HIE,[2] and approximately 25% of these infants exhibit permanent motor and/or cognitive disability.[3–5] Encephalopathy occurs when blood supply to the brain is interrupted leading to diminished delivery of oxygen and nutrients to the tissues.[6] Thus, newborns with encephalopathy can present with respiratory distress, altered levels of consciousness, changes in tone, and seizures.[2] Therapeutic strategies have focused on supportive care and the use of steroids and allopurinol to reduce the extent of cerebral edema and oxidant injury. However, these approaches do not provide adequate neuroprotection, and long-term neurodevelopmental outcomes remain poor.[7–9] Controlled hypothermia is currently the only therapy that has been shown to significantly improve outcomes, and it is the standard of care for term infants with HIE.[10,11] Animal studies have demonstrated that hypothermia protects the brain from neuronal damage and cell death after ischemia, decreasing histologic evidence of injury and improving neurological and behavioral outcomes.[9] However, hypothermia is associated with some inherent risks, must be initiated within six hours after injury, and is more efficacious for those with mild and moderate rather than severe HIE.[12,13]

Previous studies have suggested that bone marrow-derived mesenchymal stem cells may reduce injury in animal models of cerebral ischemia, possibly due to their anti-inflammatory and anti-apoptotic properties,[6,14,15] as well as

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amniotic stem cells[16] and embryonic stem cells.[17] Nevertheless, the use of bone marrow cells presents significant technical hurdles. Bone marrow aspiration is an invasive procedure, and the yield of aspirates is generally low and further reduced with age and co-morbidities.[18,19] Human adipose stem cells (hASCs) represent an alternative source of mesenchymal stem cells that is abundant, accessible, easily harvested, and unaffected by age or disease.[6,16,20–23] hASCs mobilize to areas of injury, where they suppress inflammatory gene expression, induce angiogenesis, and differentiate into neurons and other cell types.[24,25] In addition, in previous studies there were increased survival rates in rats with HIE and improved learning and memory after transplantation of hASC.[26,27] There have been studies evaluating autologous versus allogenic adipose derived stem cells that noted decreased apoptosis of neuronal cells and improved neurological outcomes.[22,23,28] In the present study, we determined whether intravenously (IV) administered hASCs provide long term neurodevelopmental protection in an experimental model of HIE in neonatal rat pups.

MATERIALS AND METHODS

This study was carried out in strict accordance with the recommendations in the Guide for the Care and Use of Laboratory Animals of the National Institutes of Health. All protocols were approved by the Institutional Animal Care and Use Committee, at Rutgers Robert Wood Johnson Medical School and Cooper University Hospital institutional review board. All surgery was performed under isoflurane anesthesia, and all efforts were made to minimize suffering. Animal health and behavior were monitored frequently during surgery and initial procedures, and then daily for the duration of the experiment. All animal welfare considerations were taken, including efforts to minimize suffering and distress, use of analgesics or anesthetics, and special housing conditions. Humane endpoint was considered. However, due to the need to evaluate brain tissue, euthanasia was required as the endpoint of the experiment. Total length of the experiment was 42 days, and animals were euthanized between day 43 and 49 of life.

A total of 84 animals were used, of these 62 reached completion of the experiment and 22 died prior to the completion of the experiment. Of the animals that died, 4 did not survive surgery and 3 died during the period of hypoxia. Remainder of the animals were cannibalized or died of alternate causes.

Animal health and behavior were monitored daily by the research staff or vivarium staff. All animal welfare considerations were taken to minimize the suffering and distress of the animals. This included, but was not limited to allowing the rat pups appropriate time with the dam until they were weaned off, use of nestlets as enrichment material, use of analgesia (Buprenorphine) and anesthesia (inhaled isoflurane) during surgery or any painful procedure. All animals were housed in polycarbonate shoe box with microisolater lid.

Institutional animal ethics committee specifically reviewed and approved the anticipated mortality in the study design, as it was similar to other similar research studies.

FAT HARVEST

Cooper University Hospital institutional review board granted approval to obtain fresh human adipose tissue from adult patients who underwent liposuction after informed consent was obtained.[29] Tumescence solution (25-50 mL) was infiltrated through an incision in the targeted liposuction sites, and 50 mL of lipo-aspirate obtained using a 3 mm Mercedes cannula.

STEM CELL ISOLATION

Lipo-aspirates were centrifuged, and fat, blood and tumescent fluid layers discarded. The stromal vascular layer containing stem cells was suspended in medium consisting of M199 at 37°C and plated on T25 plastic cell culture flasks (Falcon). These cells were trypsinized and washed three times in PBS yielding approximately 10^8 cells, which were used for injection.

HIE MODEL

One of the commonly used pre-clinical HIE models consists of a permanent unilateral common carotid artery ligation followed by a period of systemic hypoxia produced by inhalation of 8% oxygen/balance nitrogen for various periods of time at a controlled temperature. The ligation of one carotid artery in itself does not affect cerebral blood flow (CBF) to either hemisphere, because of the Circle of Willis in the vasculature of the rat brain. However, during the hypoxic exposure, the rats become hypotensive, with a fall in mean blood pressure of 25–30%, with a corresponding fall in CBF in the hemisphere ipsilateral to the ligation of 40–60% of control.[3] CBF returns to normal immediately upon return to normoxia and reperfusion. The brain damage that results is normally confined to the ipsilateral hemisphere and can range from mild selective neuronal necrosis to severe infarction, depending largely on the duration of the hypoxic–ischaemic interval, although there is animal-to-animal variability in the model.[30] In our model, seven day old Sprague–Dawley rat pups were anesthetized with isoflurane. The left common carotid artery was either isolated in control animals (sham group) or ligated (HIE group) via a midline neck incision, as described by Rice, Vannucci, et al.[3,21,31] All pups were returned to the dam immediately after surgery for a recuperation time of 1-2 hours. Pups in the experimental group were then placed in a sealed container and exposed to 8% oxygen/92% nitrogen at 36°C for 120 min.

STEM CELL INFUSION

At 48-72 hours after surgery, half of each group (sham, HIE) received intravenous hASCs (0.5-1 x 10^6 in 0.1 mL of normal saline) and half received NS control (0.1 mL) by injection via the tail vein ([Figure 1](#)).
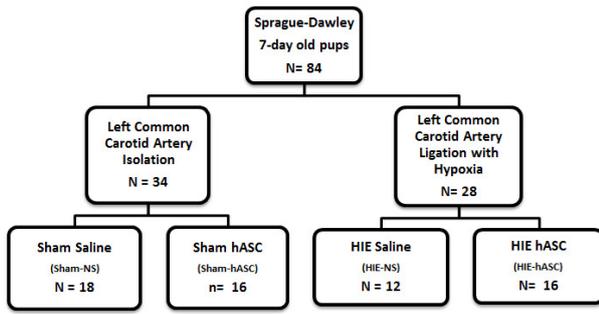
Figure 1. Flow Diagram

Total number of animals included in the study with ~74% overall survival. Mortality rate was a result of surgery (19%), HI (14%), and other causes (67%).
For abbreviations, see text.

BEHAVIORAL TESTS

Accelerating cylinder and Rota-rod (IITC Life Science Rota-rod for mice and rats) testing was performed at weeks 2, 4 and 6 of life. Cylinder tests assessed asymmetry of the forelimb and movements during exploratory activity within a 15 cm by 30 cm transparent cylinder.[14,32–35] A mirror was placed behind the cylinder and each animal was video recorded and observed for 3 minutes. The number of touches of the walls of the cylinder with each of the front paws was recorded.[12–14] Paw preference was calculated by [(left forepaw – right forepaw)/ (left forepaw + right forepaw+ both)] * 100. The evaluators of the videos were blinded to the treatment group.

Rota-rod testing assesses skill learning, balance, and motor coordination. Rats were placed on a rotating rod at a starting speed of 5 revolutions per minute (rpm), which steadily accelerated to a maximum of 45 rpm over 30 seconds. Testing continued until the animal fell off the cylinder or gripped it for two full revolutions. Time, speed, and distance were recorded during three separate trials 15 minutes apart and the results averaged for analysis.[12–14]

Repeated measures ANOVA (generalized linear model) was used to compare most of the continuous variables. One Way ANOVA with post-hoc Tukey testing was performed to compare performance over time. Categorical data was analyzed using Chi Square Test where appropriate. Statistical significance was considered when $p<0.05$.

CORTICAL THICKNESS

Six weeks after injection, rats underwent euthanasia using isoflurane. Brains were perfused with NS and 4% paraformeldahyde. Brain tissue was removed and four equal-sized coronal sections were cut, fixed, and embedded in paraffin. Sections of midbrain (10 μm) were stained using Nissl staining with Cresyl Violet and cortical thickness measured. Neuronal survival and hemispheric volume were compared in the four experimental groups and between lesioned and contralateral hemispheres of the HIE animals using Image J software (ImageJ 1.48).

RESULTS

BODY WEIGHT

Total of 84 Sprague-Dawley 7D rat pups were included (Fig. 1). Although initial body weights were similar in all groups (p=0.76) prior to surgery, weight decreased after surgery (2 weeks of life) in the HIE with normal saline (HIE-NS, p= 0.07) and HIE with human adipose stem cell (HIE-hASC, p= 0.03) groups. At 4 weeks of life, body weight in the HIE-NS group remained significantly lower than the sham groups (p= 0.005), while the HIE-hASC group became similar to shams (p= 0.18). Six weeks after surgery, there was no significant weight differences between groups (p=0.88) (Table 1, Fig. 2). Total number of animals included in the study, with ~74% overall survival. All of the above calculations were performed using ANOVA with post hoc analysis.

CYLINDER TESTING

Movement of the right paw, contralateral to the side of carotid artery isolation or ligation, was assessed to determine the severity of motor injury. ANOVA with Tukey post hoc analysis was used to compare groups. There were no statistically significant differences in the use of the right paw in cylinder testing between groups at weeks two and four. During week six, the HIE-hASC group demonstrated increased use of right paw compared to the HIE-NS group (p=0.05), and continued to be similar to the sham groups (p= 0.78) (Fig. 3).

ROTA-ROD TESTING

Kruskal Wallis and Mann Whitney U Test were used for analysis and post hoc analysis of Rota-rod performance, respectively. There were no statistically significant differences in the distance travelled on the Rota-rod between the groups during weeks 2 and 4. However, during the sixth week the HIE-hASC group had improved performance on the Rota-rod (p=0.03) compared to the sham and HIE-NS groups. (Fig. 4)

CORTICAL THICKNESS

ANOVA and Student T test were used to compare difference in cortical thickness between groups. There was significant atrophy in the lesioned hemisphere of the HIE groups compared to the contralateral hemispheres and to shams. Sham-NS and sham-hASC animals had 97.8% and 99.4% cortical sparing, respectively. HIE-NS group had significantly less cortical sparing in the lesioned hemisphere (75.6%) compared to the sham-NS and sham-hASC groups (p=0.04 and 0.03 respectively). HIE-hASC animals had improved cortical sparing at 92.3% compared to HIE-NS group (p=0.1), and was still significantly different from the sham groups (p= 0.02 and 0.03 respectively) (Fig. 5).

Table 1. Demographics

	Sham Saline	Sham Cells	Ligation Saline	Ligation Cells
Male Sex (%)	28%	44%	50%	44%
Birth Weight, grams, SD	1.5 ± 0.2	1.5 ± 0.2	1.5 ± 0.1	1.5 ± 0.1
Weight Day 12, grams, SD	26. 3 ± 4.5	24.3 ± 3.2	22.3 ± 3.6	22.3 ± 3.8
Weight Day 28, grams, SD	60.1 ± 8.5	57.7 ± 4.5	52.3 ± 7.4	55.4 ± 5.6
Weight Day 42, grams, SD	130.0 ± 17.8	129.5 ± 15.8	127.9 ± 17.5	125.6 ±17.1

Sham – control animals, Ligations – animals who underwent hypoxic-ischemic insult, Saline - control animals who received normal saline, and Cells – animals who received hASC's.
All the weights are listed in grams ± standard deviation.

Figure 2. Changes in body weight over time

Legend: ◆ Represents sham-NS , ■ represents sham-hASC, ◆ represents HIE-NS
◆ represents HIE-hASC. *p<0.05
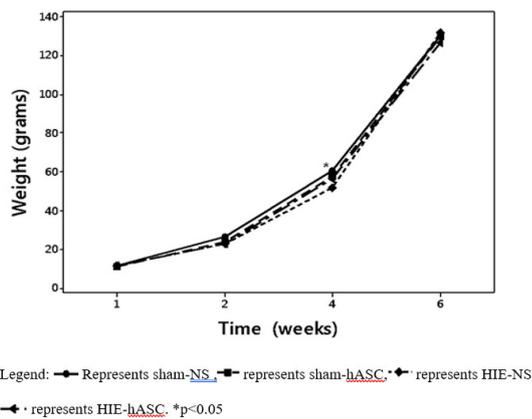
Figure 2. Changes in body weight over time

Weight was the same for all 4 groups prior to surgery, with decrease after surgery in the HIE-NS (p= 0.07) and HIE-hASC (p= 0.03) groups. At 4 weeks, weight in the HIE-NS group remained significantly lower than the sham groups (p= 0.005), while the HIE-hASC group became similar to shams (p= 0.18). After six weeks there was no differences between groups (p=0.88).

Legend: ◆ Represents HIE-hASc, ■ represents HIE-NS, ◆ represents Sham-hASc and,
▲ represents Sham-NS. *p<0.05

Figure 3. Cylinder test

Percent use of unaffected (contralateral) compared to affected (ipsilateral) forelimb during explatory activity in a cylinder after HIE (weeks 2-6). HIE-hASC group had increased use of right paw compared to the HIE-NS group (p=0.05), similar to the sham groups (p= 0.78).

Figure 4. Rota-rod testing over time

Legend: ◆ Represents HIE-HASc, ■ represents HIE-NS, ◆ represents Sham-hASc, and ,
▲ represents Sham-NS.

Figure 4. Rota-rod testing over time

Sensory motor function and learning assessed using a rotating treadmill. The HIE-hASC group traveled further on the Rota Rod (p=0.03) compared to the sham and HIE-NS groups in week 6.
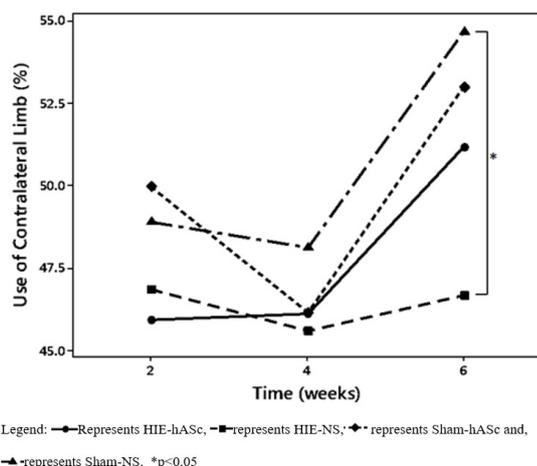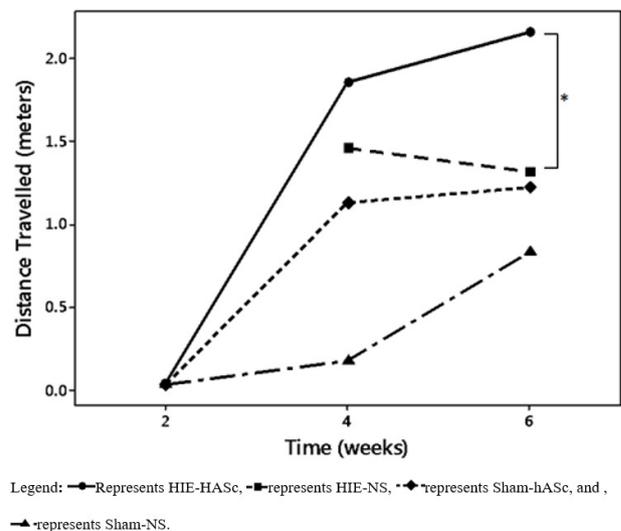
DISCUSSION

Human adipose tissue is an abundant source of mesenchymal stem cells, which are fibroblast-like cells that are easily harvested, can be expanded easily in vitro, are homogeneous, and have multi-lineage potential.[9] In 2001, Zuk and colleagues found that hASCs from lipoaspirates could be isolated and grown in large quantities for potential therapeutic use.[9,36] hASCs are increasingly being used in research as a treatment or adjunct therapy for brain injury.[4,24,27] These cells have anti-inflammatory and immunomodulatory properties that minimize host rejection and allow them to be used in tissue engineering.[6] Kim and colleagues showed that transplantation of hASC into adult rats with experimentally-induced stroke reduced cerebral inflammation and cerebral degeneration.[37]

We found that IV injection of hASCs is feasible in neonatal rats. In contrast to previous studies that delivered hASCs to rats via intraventricular, intranasal, or intraperitoneal route, we injected hASCs into a peripheral vein to closer resemble the most likely route of access for affected
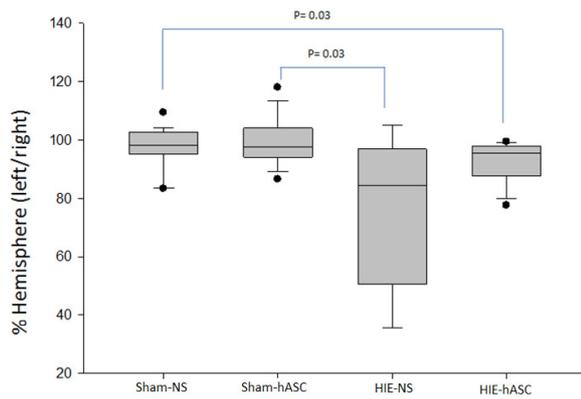
Figure 5. Cortical hemispheric volume

Percentage of hemispheric volume of lesioned versus contralateral hemispheres was compared between the four experimental groups. The left hemisphere became most atrophic after HIE and NS, with cortical volume decrease to 75% of the right side (p=0.03). After hASC injection the ipsilateral hemispheric atrophy was much less with decrease to 92% left cortical thickness compared to right. Data presented as mean±SD and CI. For abbreviations, see text.

neonates.[38,39] Similarly, Yang [27], Chen [40], and Yashura (2006) demonstrated that intravenous administration of multipotent cells in injured animals resulted in implantation of the cells at sites of injury with significant improvements in neurodevelopmental outcomes.[27,38,40] In our study, hASCs improved neurodevelopmental outcomes, suggesting that they are viable and biologically active in the brain when delivered peripherally.

Two established neurodevelopmental tests were used to assess cognitive development. The Rota-rod and cylinder tests have been validated to quantify distinct aspects of sensorimotor deficits caused by cortical and striatal injuries.[37] Due to equipment malfunctioning we were unable to perform Rota-rod testing of the HIE-NS animals in week 2. Improved performance on Rota-rod testing was noted in week 4 after IV hASC injection, when compared to sham-NS animals. It was also noted that those rats in the HIE-hASC group showed greater use of the affected limb in week 6 when compared to shams and NS controls. The use of the contralateral limb was similar in all groups during the first four weeks of life. However, rats in the HIE-hASC group had improved functioning of the affected limb in week 6. These findings suggest that hASCs can preserve neural tissue and improve long term neurodevelopmental outcomes after HIE. Our findings of improved neurodevelopmental outcomes with use of hASC are similar to those by Park et al.,[41] who reported that injection of human adipose cells into cerebral ventricles in rats after HIE resulted in im-

proved physical activity for as long as 7 weeks post-injection.[41]

We found that administration of hASCs tended to preserve cortical volume after HIE. Atrophy was very evident in the HIE-NS group; however, there was only a minor difference in cortical thickness between sham and HIE-hASC. There was higher statistical significance than expected possibly due to a difference in sample size between groups. A previous study by Van Velthoven et al and Lee et al reported that hemisphere size was not fully restored after infusion of human mesenchymal stem cells.[6,14] However, in our study when comparing the cortical thickness of the HIE-hASC group with that of the sham groups there was a significant preservation of hemisphere size. This finding implies that the IV hASCs likely migrated to the area of injury and exerted a regenerative or protective response.

Six week old SD pups were chosen in this experiment to demonstrate long-term neurodevelopment. Sengupta's paper on relating rat ages to human years showed that rats reach their pubertal stage at approximately 38 days which correlates to a human age of 11 to 12 years.[42] This allows us to correlate the neurodevelopment outcomes from birth to puberty.

Our study has several limitations. We did not label the stem cells prior to injections and relied on the assumptions from previous research that hASCs can traverse the blood brain barrier. Stem cells tend to migrate to areas of inflammation, and so could have been diverted to other sites of hypoxic-ischemic injury or areas of inflammation. However, our positive findings on functional neurodevelopmental testing and preservation of cortical thickness suggest that the hASCs exerted cognitive and biologic effects on the brain. In addition, the experiment was not executed to determine whether these effects are dependent on implantation and differentiation of the hASCs, or whether soluble products of these cells mediate these changes. It is also possible that negative findings are a result of Type II error related to our limited sample size.

In conclusion, we found that IV injection of hASCs is a feasible and effective method for stem cell administration. Treatment of neonatal rats with hASCs post-HIE was associated with improvement in balance and coordination, and possibly with improvement in affected limb usage. Rats receiving hASCs had less cortical atrophy similar to sham animals when compared to NS controls. Further studies are required to characterize the nature, extent, and mechanisms of the effects of hASCs in the treatment of HIE.

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